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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This paper presents the first use of preparative isotachophoresis to isolate PA from the crude whole toxin. The distinct roles of PA and LF were first demonstrated by Beall et al. (ref. 2) and subsequently their usefulness as potential immunogens was confirmed by Mahlandt et al. (ref. 4). In addition to having shown the serologic specificity of PA and LF derived by preparative isotachophoresis (Fig. 1, and Fig. 2), we subsequently demonstrated that homologous antibodies to PA and LF when added individually to identical aliquots

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of whole crude-toxin blocked lethality following intravenous injection into susceptible Fischer 344 rats. The potential use of preparative isotachophoresis in improving vaccine production is still being evaluated, as is the biological integrity of the individually recognizable components.

ISOLATION OF PROTECTIVE ANTIGEN FROM ANTHRAX TOXIN BY PREPARATIVE ISOTACHOPHORESIS

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INTRODUCTION

Anthrax is a disease of great historical and current interest. The bacillus was first observed by Davaine and Rayner in 1850. In 1857 Brauell transmitted the disease by the inoculation of blood from infected animals. However it was Koch, who in 1877 cultured the bacillus in the aqueous humor of an ox's eye, and demonstrated the causal relationship between the organism and the disease.

Students of microbiology are immediately impressed with a number of important facts about anthrax. The first is that anthrax, like plague, Legionnaires' disease or Rocky Mountain spotted fever is a disease which if undetected and untreated will progress to a temporal point of almost certain lethality even if appropriate antibiotics are then belatedly given. Students also learn that the organism has a capsule of polymerized D-glutamic acid which inhibits phagocytosis and allows the bacillus to establish an infectious focus and elaborate a tripartite toxin. This unique toxin complex has components which the British have termed factors I, II and III (ref. 1) and the Americans have more descriptively called edema factor (EF), protective antigen (PA) and lethal factor (LF) (ref. 2). The pivotal role is played by PA, for without it EF does not cause edema and LF is not lethal.

As the term implies protective antigen serves as the key component in protective vaccines against anthrax. In the past, separation of the individual toxin components relied on adsorption on glass filters or hydroxyapatite and/or anion exchange chromatography. This paper presents the first use of preparative isotachophoresis to isolate PA from the crude whole toxin.

METHODS

Toxin production

Anthrax toxin was produced from either the Vollum 1B or Sterne strains of Bacillus anthracis in a chemically defined media using the method of Ristroph and Ivins (ref. 3).

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Production of antisera

Initially, antiserum to the three toxin components was made using 0.5 ml of Jen-Sal (anthrax spore vaccine, Jensen-Salsbery Labs, Kansas City, MO, #131382) given by intramuscular injections into goats at 2-week intervals and bled at appropriate times. In a similar fashion antiserum to PA was made using a PA immunogen produced by the Michigan Department of Public Health, Lansing, MI. Affinity chromatography was subsequently used to remove any cross-reactivity.

Affinity chromatography

Protein-A-sepharose CL-4B (Pharmacia) was used to bind IgG antibodies of known specificity. Whole toxin or isolated components could be removed selectively. See results section.

Preparative isotachophoresis

A preparative apparatus (uniphor 7900 LKB, Bromma, Sweden) was used employing a 4.5% polyacrylamide gel supporting medium. Single buffered gel columns (cross-sectional area, 3.48 cm^2, length, 14 cm) were used. De-aerated Tris-phosphate (pH 7.03) was used in both the lower electrode (anode) chamber and the elution buffer. The terminating buffer Tris-ε-aminocaproate (Tris-EACA), pH 8.4, was used in the upper electrode (cathode) chamber and in the column. Fifteen ml of toxin previously assayed by intravenous injection into Fischer 344 rats, were dialyzed for 18 hr against normal saline at 4°C.

This sample was put through a 0.22-μ filter; 0.5 ml of carrier ampholytes pH 3.5 to 9 (ampholine LKB) and 1 ml glycerol were added. The mixture was then layered on top of the gel through tubing inserted beneath the top layer of Tris-EACA buffer and held within a few mm of the gel surface. Using a LKB 2127 constant power source a current of 6 mA and a starting voltage of 1.8 kV were applied in each run. Cooling water was ambient and ranged between 20-28°C. The elution rate was 18 ml/hr. Three-ml fractions were collected and assayed at UV absorption of 280 nm on a Beckman DBG spectrophotometer.

RESULTS

Fig. 1 demonstrates the specificity of the goat antiserum to protective antigen (PA). Protective antigen derived from either the Michigan Department of Public Health and the preparative isotachophor separations gave single lines of common identity.

Note that while crude toxin (CT) contains all three components and would be expected to contain one identical line with PA, lethal factor (LF) and edema factor (EF) are not recognized by the goat anti-PA antibodies. Likewise in Fig. 2, anti-LF antibodies recognize only LF and the LF component of crude toxin (CT) but not PA or EF.

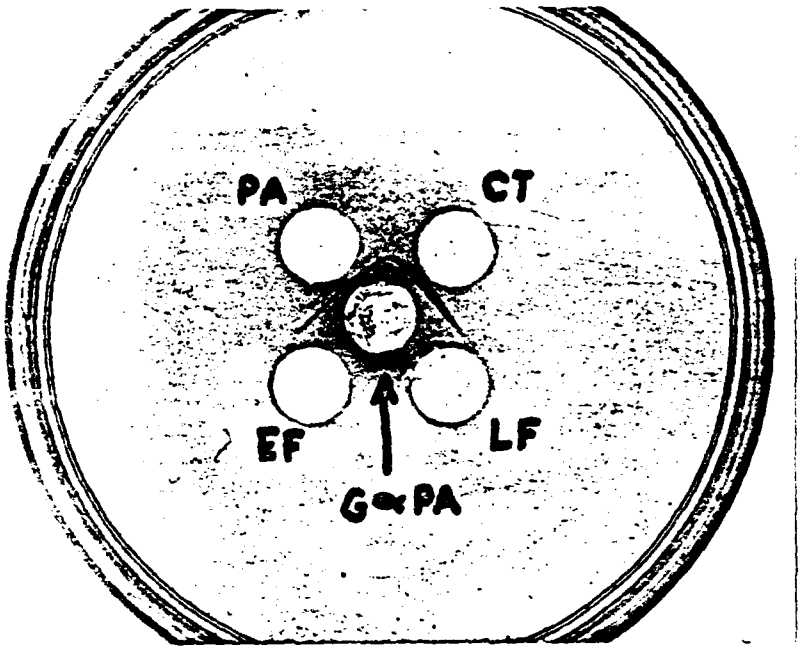

Fig. 1. Specifity of antiserum to protective antigen. There is a single line of identity with isolated PA and PA found in whole CT.

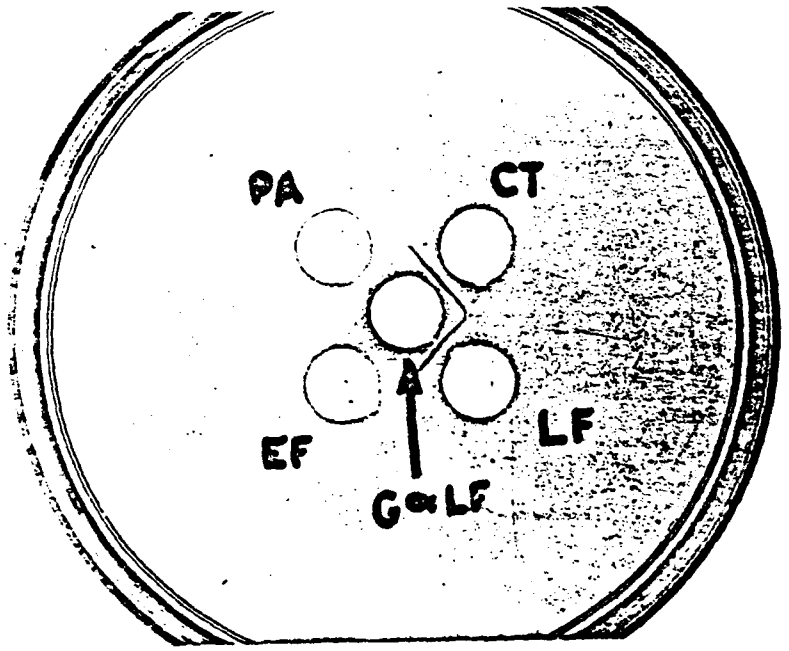

Fig. 2. Specificity of antiserum to lethal factor. There is a single line of identity with isolated LF and LF found in whole CT.

Fig. 3 demonstrates a characteristic preparative run of whole crude toxin concentrated with polyethylene glycol and dialyzed against normal saline.

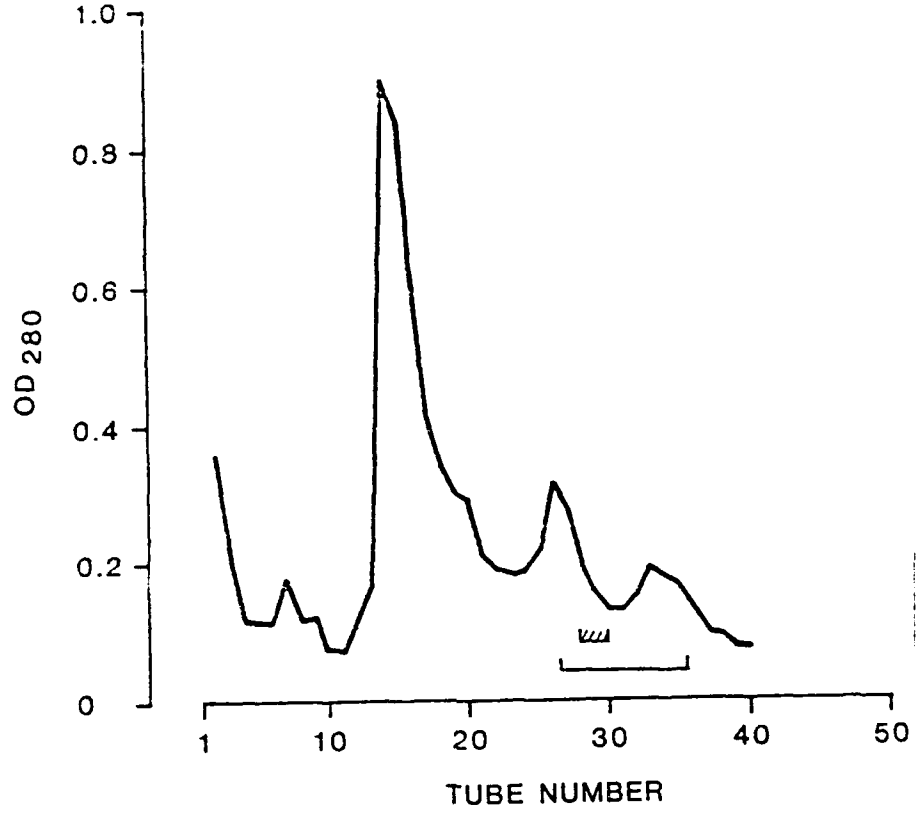

Fig. 3. Preparative isotachophoresis run from Uniphor column in which whole crude toxin was separated. LF and PA extend from fractions 27 to 36. PA, present in open brackets, extends from tubes 27 to 36. LF, in cross-hatched area, extends from tubes 28 to 30.

Fig. 4 shows the individual isotachophoretic fractions run against goat anti-PA antibodies. PA is found in tubes 27 to 36.

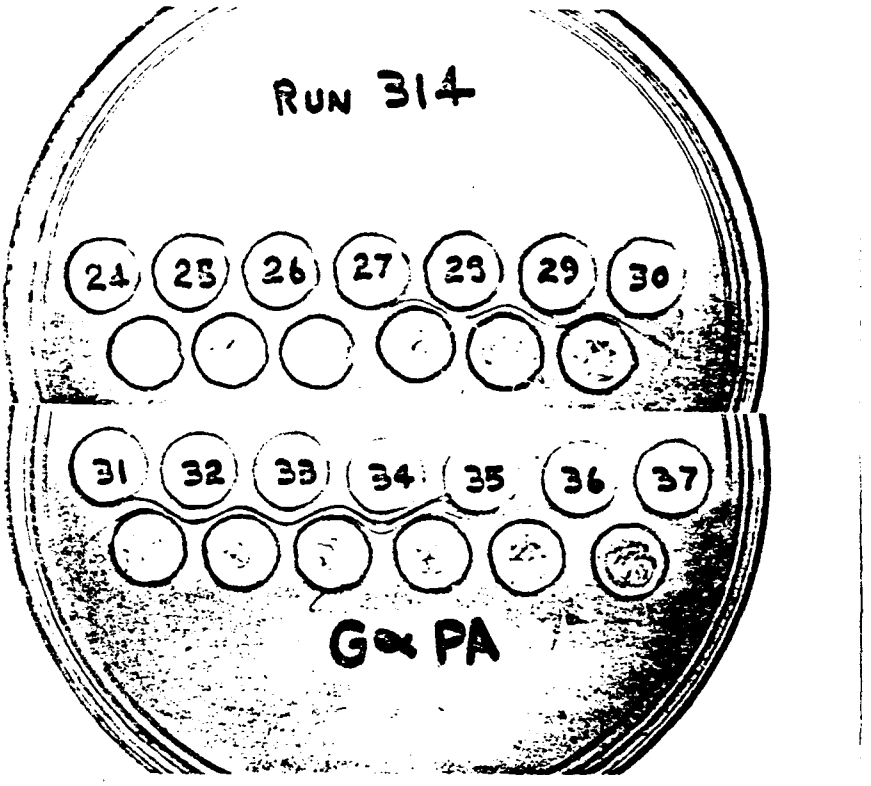

Fig. 4. The location of protective antigen by means of specific antibody in the individual fractions following isotachophoretic separation.

Fig. 5 shows the same fractions run against goat anti-LF antibodies. Here LF is limited to tubes 28 to 30.

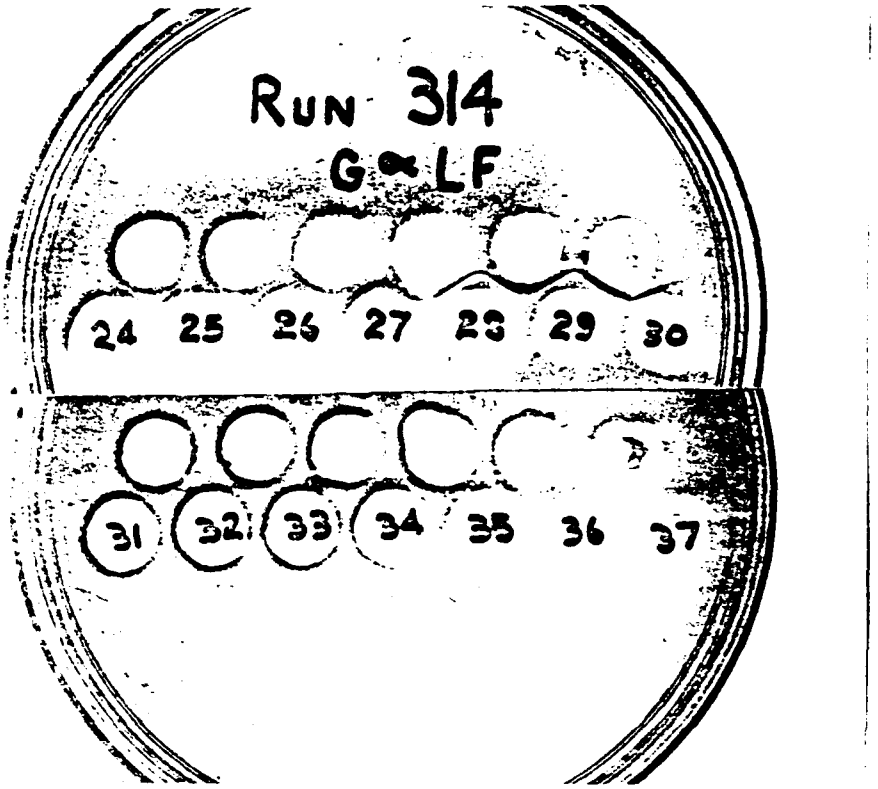

Fig. 5. The localization of lethal factor by means of specific antibody in individual fractions following isotachophoretic separation.

Fig. 6 demonstrates that if one couples anti-PA antibodies to an insoluble matrix and does affinity chromatography using insolubilized protein-A, one can remove the trace PA and isolate LF from fractions 28 and 29.

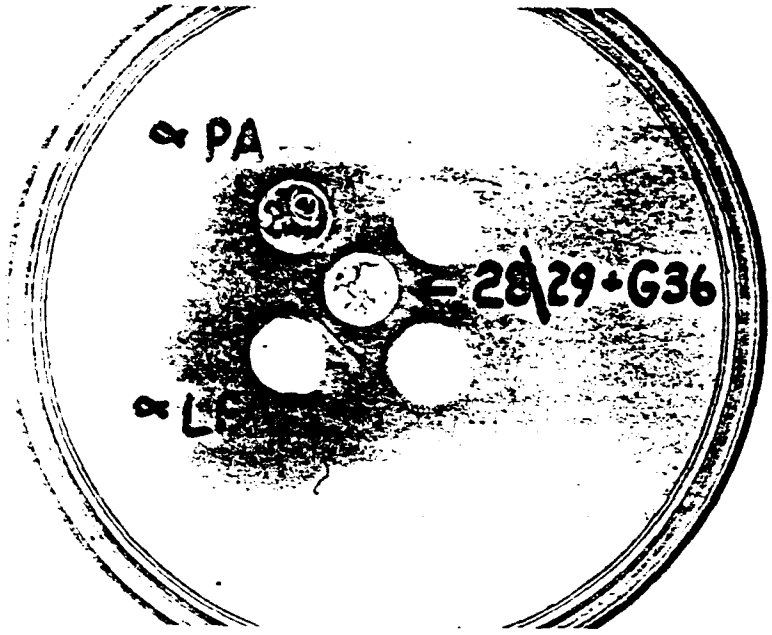

Fig. 6. Trace PA removed from fractions 28 and 29 by affinity chromatography, leaving LF alone.

Fig. 7 is simply a standard polyacrylamide 7.5%, 20-cm slab gel run for 18 hr at starting current 9 mA at 200 volts. Columns A and F are the high and low molecular weight standards. Column B is the Michigan Department of Public Health PA, column C is whole CT, columns D and E are the preparative isotachophor derived PA and LF. Their molecular weights are approximately 88,000 and 87,000 daltons.

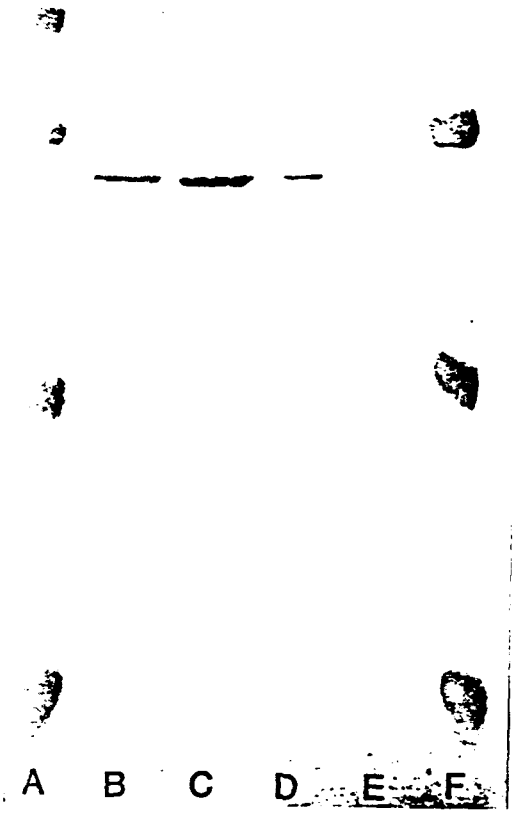

Fig. 7. Polyacrylamide gel localization of PA and LF (see text).

DISCUSSION

The distinct roles of PA and LF were first demonstrated by Beall et al. (ref. 2) and subsequently their usefulness as potential immunogens was confirmed by Mahlandt et al. (ref. 4). In addition to having shown the serologic specificity of PA and LF derived by preparative isotachophoresis (Fig. 1, and Fig. 2), we subsequently demonstrated that homologous antibodies to PA and LF when added individually to identical aliquots of whole crude-toxin blocked lethality following intravenous injection into susceptible Fischer 344 rats. The potential use of preparative isotachophoresis in improving vaccine production is still being evaluated, as is the biological integrity of the individually recognizable components.

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